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Microdissection: insights and progress in the era of precision medicine

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Abstract

Intratumoral heterogeneity is a real challenge for understanding the key mechanisms involved in cancer progression, but also for pathologists to make a reliable diagnosis. The development of precision medicine in oncology allows each treatment to be tailored to the specific characteristics of the tumours. However, the ability to isolate pure cell populations is a very difficult task and makes accurate analysis difficult. The development since the 90's of precise dissection techniques, known as microdissection, has helped to overcome problems associated with tumor heterogeneity. Since then, a few techniques have been developed that provide dissection accuracy down to the sub-cellular level. These technologies have enabled key principles to be understood in basic research but handling difficulties have prevented their use in pathology laboratories. Working with microquantities can also be a difficult task and requires several technical adaptations. This review provides an overview of all microdissection techniques currently available. In addition to their main advantages, examples of applications and adaptations of molecular biology techniques to microquantities are proposed to illustrate the interest of these technical approaches in both basic research and clinical applications.

Keywords:

Microdissection, cancer, tumour heterogeneity, FFPE, precision medicine

1. Introduction

Making an accurate and reliable diagnosis is a daily challenge for pathologists. Initially based on the macroscopic and histological analysis of tumours, diagnosis has been coupled with molecular analysis in the last decade[1,2]. The high level of cellular diversity within tumours implies the presence of varied genetic, transcriptomic and proteomic profiles[3,4]. The ability to isolate and analyse specific subpopulations within tumours is therefore of real interest, both for

improving patient care[5,6] but also for a better understanding of the underlying mechanism driving tumour evolution. To respond the challenges posed by tissue heterogeneity, microdissection techniques, which refer to all dissection techniques using a microscopic approach, have been developed. At first performed manually, microdissection really gained in popularity at the end of the 20th century, with the development of laser microdissection by Emmert Buck et al[7]. Laser microdissection has contributed greatly to elucidating the complex mechanisms underlying pathologies such as cancer[8,9] or other biological fields[10–12] by allowing the tissue dissection to reach a subcellular level. The price and complex handling represent a limitation within these technics for development and daily used for diagnostic purposes. However, the pathologist's need for access to precise and routinely usable microdissection platforms regularly increases with the advent of sensitive molecular diagnoses. This led to the development of a new generation of innovative microdissection systems, which have made it possible, step by step, to widely use accurate microdissection in pathology laboratories. Microdissection can be performed on fresh frozen (FF) or formalin-fixed paraffin embedded (FFPE) tissues. Nevertheless, FFPE tissues are privileged for diagnoses because of the low cost of storage and their accurate long term preservation[13]. FFPE samples stored in large quantities over the world are an invaluable resource for studying the key molecular mechanisms underlying disease progression[14].

The present review gives an overview of all the techniques identified as microdissection and currently used to study FFPE tissues. A specific focus on the progress made possible by microdissection in the cancer field is proposed to illustrate the benefits offered by these technologies, both in terms of basic research and clinical diagnosis. The challenge and adaptations needed for omics techniques by working with microquantities of FFPE samples will be presented and discussed.

2. Microdissection techniques

2.1. Manual microdissection

Manual microdissection was the first and for a long time the only microdissection technique used in research even no publication can attest this affirmation. Indeed, until the early 20th century, there was no truly effective microdissection technology available. In this context, all research and diagnostic laboratories wishing to isolate regions of interest had to perform this task manually or using homemade technologies that were not documented in the literature. This technique consists in isolating the tissue region of interest (ROI) usually by manually scraping under standard or inverted microscopy and collecting into a dedicated tube for subsequent analysis. Stereomicroscopes, which offer more space for handling, are also frequently used. The scraped area is then identified by superposition with the stained slide annotated by the pathologist[15]. Various tools such as scalpels, fine needles or ultrasonic needles are used to carry out the manipulation [16–20].

The low associated cost and ease of use make manual microdissection widely used in many laboratories. However, its cutting precision is relatively low (at the mm level), the technique is operator-dependent, and the difficulty of tracing the manipulations performed manually is also a major limitation. Consequently, isolating pure regions of interest is extremely difficult. This technique is closely related to manual macrodissection, a method that is commonly employed by pathology laboratories. The main difference is that a microscope is not used during the isolation step. As relatively large tissue areas are removed, manual macrodissection can achieve a higher throughput than manual microdissection.

2.2. Laser microdissection

In the 60's, Tomberg work described the first use of lasers as powerful tools for tissue dissection[21,22]**.** In parallel, the development of the nitrogen laser made it possible to isolate

various tissue fragments in a reliable and reproducible way with limited tissue damage[23]. Together, these findings led to the development of the very first laser microdissection integrated system[23,24]. Although precise and reliable, the difficulty of access to this technique and the absence of an effective fragment isolation system have been an obstacle to the wider development of this innovative technique. In the 90s, a new generation of laser microdissectors that made possible automatic capture of the selected area were launched with the development of molecular techniques and the increased need for access to information from complex tissues. Laser microdissection technologies employed two types of lasers, infrared laser (IR) used to capture the sample, and ultraviolet laser (UV) used to isolate the sample by cutting.

2.2.1 Laser capture microdissection

Laser Capture Microdissection (LCM) was introduced in 1996 by Emmert Buck et al.[7,] . Based on the use of an IR beams, this system can capture the ROI by using a special capsule called "Caps" coated with a thermolabile membrane made of ethylene vinyl acetate[25]. The Caps is first positioned above the slide-mounted tissue section (Figure 1.A). Under the effect of the IR beam, the membrane fuses locally with the selected area with a pre-established diameter of 7.5, 15 or 30 µm. Then, the adhesion forces between the tissue and the membrane make it possible to detach the target area with a movement of the Caps which can be used for subsequent analyses. Following these publications, the LCM technology was rapidly commercialised by Arcturus PixCell II system in 1997.

A major new development was introduced in 2004 with the launch of the Veritas by Arcturus Veritas, the first system combining UV and IR lasers thus making it possible to harvest larger ROI. Here, the system first cuts out the tissue with the UV ray (Figure 1.B). From there, the IR ray fuses the Caps membrane with the tissue to create various attachment points and then lifts it. Subsequently, several improvements were made with new versions until the Accuva Cellect

now commercialised by Laxco. In 2020, another adapted and innovative LCM system was commercialised by Fluidigm, the Acculift LCM.

2.2.2. Laser microdissection and pressure catapulting

Laser microdissection and pressure catapulting (LMPC system) technology was initially introduced by Schütze K. in the 90's[26,27,28]**.** This technology is now used by Zeiss as the PALM MicroBeam system. LMPC technology combines the use of a microbeam UV laser and a laser pressure catapulting system in a motorised inverted microscope. Here, the isolation is contact free, thus reducing the risk of sample contamination. The selected area is first cut by a UV ray (Figure 1.C) and then catapulted from the slide into the microfuge tubes by a high-energy single pulse of a defocused UV ray. Samples are mounted on an energy transfer coating membrane made of polyethylene naphthalate which allows the entire ROI to be catapulted in a single stroke and thus maintain its structural integrity.

2.2.3. Laser microdissection by gravity

Initially described by Kolbe et al. in 2000[29], this technology, commercialised by Leica as the Leica LMD 6500 (fixed and soft tissues) and LMD 7000 (thick and hard tissues) is based on the use of a UV ray in an upright microscope. To make dissection possible, the sample is sandwiched between an energy transfer coating membrane made of polyphenylene sulphide or polyethylene naphthalate, which facilitates sample dropping, and a metal frame. In the first step, the ROI is cut by the UV diode laser (Figure 1.D). Then, to allow it to fall, the sample is placed upside down and falls by gravity directly after the laser cutting. The LMD system is contact free, thus reducing the risk of sample contamination. Accurate cutting precision is also made possible by the fact that there is only the laser moving during the cutting step.

2.2.4. Laser microbeam microdissection

The Cell Cut system by Molecular Machine Industry was launched in 1998. Its adaptability to a wide range of microscopes makes it easily accessible. This technology uses a low-energy beam (microbeam), which is less traumatic for the tissue. As with the Leica system, this is the laser that moves during the cutting process, providing high cutting precision. Defined as a type of LCM, the sample is here sandwiched between a polyethylene trephthalate membrane and a metal frame. The ROI is first cut out by a UV laser (Figure 1.E) followed by capture in a recovery tube, called an insulation cap, which contains an adhesive part. At the end of the process, the adhesive cap is lowered to catch the ROI and lifted to remove it.

2.2.5. Advantages and disadvantages of using lasers

Whatever isolation technique is chosen, special attention should be given to the structural damage to the sampling area and its impact on future experiments. In this context, using laser beams can result in damage to the targeted tissue. When IR rays heat the tissues significantly[7], the UV rays remove the targeted tissue by photo ablation[30,31].

IR rays are less damaging to targeted tissues than UV. Their energy, which is weaker than UV rays, is partly absorbed by thermolabile membranes. A very brief temperature variation (µs) is thus transmitted to the sample, but it is not enough to induce damage[7,32]. UV rays have a higher energy content, which allows them to cut tissue. In addition, this energy can induce degradation of the tissue adjacent to the cutting zone[31]. To reduce this risk, laser systems minimise both the power of the rays and the duration of exposure. In fact, ray intensities are in the µJ range, and exposure times vary from picoseconds (MMI) to nanoseconds (Zeiss and Leïca). Moreover, the reduced laser diameter (<µm) reduces the area affected by the laser's energy[31]. It has also been shown on the Zeiss system that thermal changes induced by UV rays are very brief and localised but can still lead to DNA damage as shown in murine liver[31].

Much of the energy transmitted by the laser is absorbed by the thermolabile membranes used[7,31] and the low energy absorbed by tissues is enough to induce DNA damage[30]. Despite this convenience, as will be demonstrated later in this review, the applicability of laser techniques with subsequent molecular analysis has been shown in a wide range of techniques.

2.3. Mesodissection

Introduced by Adey et al. in 2013[34], mesodissection refers to a variety of dissection techniques whose precision lies between laser and manual microdissection. Mesodissection techniques respond to a growing need for accessible and rapid dissection tools for routine use. Since then, a great many technologies identified as mesodissection, offering variable cutting resolutions, have been developed.

2.3.1. Milling and microfluidics

An innovative mesodissection platform, combining a milling and a microfluidic system, was described by AvanSci Bio in 2013[34,35]. Here, a milling machine is fitted with a razor blade which has a size ranging from 100 µm to 1,200 µm. The milling machine is also coupled with a syringe that contains two reservoirs (Figure 2.A): i) the first (outer reservoir) contains an enzymatic digestion solution which is delivered in contact with the tissue that is progressively lysed through the combined action of the blade and the enzymatic solution; ii) the mix is finally vacuumed into the second reservoir (inner reservoir) that makes it possible to transfer it to a specific tube. This technology is operated by joystick and has a precision of 60 µm[34].

Based on the principle described above, the Avenio Millisect system, commercialised by Roche, was launched in 2017. This instrument contains a milling module composed of fluidic reservoirs and a blade. Here, the milling blades have three different sizes (250 µm, 525 µm and 725 µm) adapted to the size of the ROI. The lysed tissue is collected in the milling module after

dissection and deposited in a collection tube for downstream application as described below[36]. After identification of the ROI, the Avenio Millisect system will automatically align the annotated area on a stained slide with the corresponding unstained slides and in this way transfer it to the annotated area.

2.3.2. Mesodissection by capillarity

Mesodissection by capillarity, developed by NDX, is based on aspiration of the ROI through a needle named "Disposable Capillary Unit" which has an inner size between 10 µm and 100 µm. Initially designed for applications on living and FF tissue, the latest models, Unipick and Unipick+, make it possible to isolate the target area directly from the FFPE sections. Here, the Disposable Capillary Unit system is positioned above the ROI (Figure 2.B) which is aspirated by capillarity and deposited into a dedicated tube for subsequent analyses. Surette et al. have modified and adapted this technology for dissection of FFPE tissue by incorporating microfluidics tools[37]. In this system, the Disposable Capillary Unit needle is replaced with a digestion chamber that contains an enzymatic solution with detergents and proteinase K. The digestion chamber has an adjustable diameter from 0.5 mm to 3 mm. In the original article, studies were carried out on colon and invasive ductal breast carcinoma and demonstrated the applicability of this system for DNA extraction and sequencing[37].

2.3.3. Mesodissection by scraping

Xyall has developed a mesodissection platform fully adapted to high analysis throughput by imitating the scraping action performed manually (https://xyall.com/). In this device, the target area is peeled off by the action of a specific blade called a scraping head with precision of less than 100 µm (Figure 2.C). The ROI is first selected by the pathologist on a digital slide and then automatically transferred to the corresponding dissected slides. Two different formats of this system have been marketed, the Tissector High Throughput in 2021 with capacity of 1,800

slides and a throughput of 80 slides per hour and the Tissector TableTop in 2022, more compact and more easily integrated into the laboratory. The Tissector TableTop system has a 72-slide capacity and a throughput of 30 slides/hour.

2.3.4. Mesodissection by coring

All of the microdissection techniques described above involve dissection directly on slides, but sampling can be also done directly from FFPE blocks (Figure 2.D).

Recently, two coring techniques were developed and commercialised, the MiniPunch proposed by Excilone (https://www.excilone.com/en/) and the TMA molmed commercialised by 3DHistech (https://www.3dhistech.com/).

The MiniPunch platform is specifically designed for molecular analysis. Samples are taken directly from the block by using a single-use needle that prevents sample cross contamination. Moreover, this system makes it possible to process up to 16 blocks in a single run with a sampling rate of less than one minute per sample. Needles have an internal diameter of 200 µm and the sampling depth is also adjustable from 200 µm and 700 µm. As for the Xyall and Roche systems, the ROI is initially selected on a digital tissue section and then automatically transferred to the original block by overlaying the block.

The TMA molmed has a dual function: making tissue micro arrays and sampling for molecular analysis with both specific needles. For molecular analyses, samples have similar characteristics to the MiniPunch. In fact, needles have an internal diameter of 200 µm and the sampling is adjustable between 300µm and 500µm. Cross-contamination is avoided by using a cleaning block between each sampling[38].

Both platforms are specifically dedicated to pathology laboratories and high throughput sampling works. Moreover, they appear very interesting for small-size samples such as

biopsies. By targeting the tumoral area, biomolecular studies with small quantities of tissue are possible, and it preserves precious samples stored in pathology laboratories.

Before developing these new devices, few studies have been carried out using Tissue Micro Arrays as a microdissection technique[39–41]. Adey et al. applied their milling platform directly to a FFPE block[34]. Although these authors demonstrated the feasibility of this operation, the opacity of the block makes accurate dissection difficult[34]. Despite this limitation, numerous works have been carried out directly on FFPE blocks by making cores with size-adapted needles[42–47]

3. The challenge of using FFPE samples

Using FFPE tissue dates back to the beginning of the 20th century, shortly after the identification of formaldehyde as a fixation agent for tissue preservation[48]. This process allows biological material to be preserved without morphological degradation at room temperature and to be analysed years after embedding.

Formalin fixation is the most critical step because it significantly impacts subsequent analyses. It causes protein-protein and protein-DNA cross-links[49–51]. This process also leads to strand breaks, resulting in extensive fragmentation of both DNA and RNA. Short fixation times result in incomplete tissue fixation, leading to enzymatic degradation and tissue morphology alteration. Conversely, prolonged fixation increases cross-link formation, thereby increasing nucleic acid fragmentation[52,53]. Extensive fragmentation can significantly impair amplification reactions, such as PCR. Specific adaptations, such as designing short amplicons, can help reduce these limitations[54,55]. Formalin causes sequence artifacts by generating uracil lesions (cytosine-to-uracil conversion) and deaminating 5-methylcytosine to thymine. These modifications can lead to erroneous clinical interpretations during sequencing and interfere with primer binding during PCR, thereby impeding amplification. Storage conditions can also have

a significant impact on the quality of the recovered biomolecules[55–60]. In addition, the various contaminants associated with using formalin and paraffin can affect the reliability of subsequent experiments[61].

Despite these limitations, using FFPE tissue for molecular purposes remains widely possible, especially for small samples such as biopsies. Thus, many applications have been adapted to bypass the limitations associated with these samples. Combining a very small amount of initial material with FFPE tissue as encountered in microdissection can make studying the underlying biological mechanism a true challenge. In the following paragraphs, potential applications for microdissection will illustrate its strong potential in oncology.

4. Applications for microdissection in oncology

4.1. Epigenetic analyses

The epigenetic regulation system shows great sensitivity to signals from its environment. The deregulation of these signals within tumours is frequent and can lead to the abnormal expression of key genes involved in the early event of carcinogenesis[62,63]. Analysing DNA methylation status using bisulfite conversion techniques combined with microdissection techniques thus makes it possible to demonstrate the involvement of the retrotransposon Line-1 as an early event in colorectal cancer (CRC)[43,64,65]. Combining these techniques also showed a direct connection between the hypermethylation state of the mismatch repair gene promoter region and the microsatellite-high (MIS-H) status of tumour cells [66], but also, early preneoplastic colony lesions[67] in CRC.

The cross links induced by the FFPE process make epigenome analysis complex. To counter this deleterious effect, numerous sequencing techniques have been adapted to FFPE conditions such as CHiP-seq[68–72], PAT-CHiP[73], FACT-seq[74], CUT&Tag[75] and ATAC-seq[76]. Some of these techniques have been combined with microdissection to highlight tumour specific epigenetic

signals[43,72] in human colorectal and glioblastoma multiform tissue[74], in prostate cancer tissue[65] or in lung adenocarcinoma and squamous carcinoma[66].

Various microdissection techniques have also been used to demonstrate the involvement of miRNAs in carcinogenesis. Their relatively small size increases their preservation in FPPE samples, explaining why they are widely used as biomarkers in oncology[78,79]. For instance, the direct link between a decrease in miR-200 expression in CRC and the presence of a migratory phenotype has been shown with coring techniques[45–47] but also with laser microdissection[80,81] by comparing the invasive and the central area of the tumour. In pancreatic ductal adenocarcinoma, microdissection techniques have revealed a decrease in the expression of miR-200 (b and c) at the invasive front compared to the tumour centre, suggesting an influence of the tumour microenvironment[82]. Other studies combining miRNA sequencing and microdissection have been conducted to identify predictive biomarkers of malignancy in melanoma cases[83].

4.2. Microdissection and genetic applications

Genetic and transcriptomic analyses are routinely used for diagnostic purposes in oncology. Adapting these analyses to FFPE microquantities makes it easier to implement dissection techniques in diagnostic laboratories.

Despite the severe degradation of nucleic acids in FFPE samples, next generation sequencing (NGS) analysis remains achievable. Different studies have thus shown a strong concordance between FFPE and fresh frozen samples in NGS analysis despite the presence of significant deamination of DNA extracted from FFPE samples[84–86]**.** Moreover, the development of numerous NGS sequencing protocols that can be used on small initial quantities of FFPE samples means that now, the vast majority of microdissected samples can be sequenced[87,88]**.** In Merkel cell carcinoma, Kader et.al. were able to use microdissected samples to perform low-

coverage WGS with just 5ng of DNA[89]. Here, the search for Copy Number Alteration showed similar results to when a larger quantity of DNA (100ng) was used[90]. This workflow has been applied to breast cancer and has shown that atypical ductal hyperplasia can be a precursor of high-grade and low-grade breast cancer. In cases of synchronous atypical ductal hyperplasia and carcinoma, this workflow highlights some cases where Copy Number Alterations were present only in the carcinoma, but also sometimes, only in the atypical ductal hyperplasia part. In a retrospective vulvar squamous cell carcinoma study, the tumour population enrichment combined with the use of targeted DNA-sequencing has helped to define this cancer as two distinct entities. In fact, it has been shown that HPV positive and HPV negative tumours have different mutational profiles. Moreover, an HPV positive specific mutation has been identified, opening the way to potential therapies[91].

Combining microdissection with whole-exome sequencing has thus shown its value in various studies. This workflow has shown the complexity of the processes involved for instance in the transition from *in situ* ductal carcinoma to invasive ductal carcinoma[92]. In colorectal carcinoma (CRC) associated with inflammatory bowel disease, comparing normal and tumour tissues revealed tumour-specific mutations. These results were then compared with previous results on sporadic colorectal carcinoma and showed genetic specific features that make it possible to distinguish CRC associated with inflammatory bowel disease from sporadic cases. In fact, KRAS and APC mutation rates were lower in these patients in contrast to the SOX9/EP300 and IL16 mutations which were higher. These findings open the way for the identification of new biomarkers[93]. Similarly, transcriptomic biomarkers have been highlighted through the direct comparison of several paired *in situ* ductal carcinoma and invasive breast carcinoma cases[94]. Microdissection can be also used to analyse tumour heterogeneity. In this context, significant intratumoral heterogeneity in glioblastoma cases has been demonstrated through the isolation of different subpopulations by combining LCM and RNA-seq[95]. Recently, investigations into

the origin of the combined small cell lung cancer were conducted using the same workflow[96]. Finally, a case report which investigated the direct clonal evolution link between idiopathic pulmonary fibrosis and lung cancer was conducted using LMD. Although common features were found between both tissue entities, the findings did not identify idiopathic pulmonary fibrosis as a precancerous lesion [97]. For specific mutations, such as RAS mutations, which are frequently found in colorectal cancer[98–101] using microdissection techniques can be a real advantage in guiding medical staff's therapeutic decisions. In an adenocarcinoma case report, after detecting the presence of three different type of RAS mutation inside the same tumour via a bulk test, the combination of LCM and DNA sequencing showed a high rate of intratumoral distribution of these mutations. Each ROI analysed had a specific rate of mutation profile, and some mutations were observed only in the adenoma part[102]. Other studies also highlighted a discrepancy in the mutational profile between adenoma and carcinoma in 16 of the 70 cases studied[103]. A further comparison of adenoma and carcinoma using microdissection also showed a discordance in mutation rate between the two entities of 35.7% across all the cases included in this study. Importantly, in some cases, the mutation was found only in the adenoma part[104]. These three studies have therefore highlighted the value of using microdissection techniques to show discrepancies between different areas of the tumour, thus helping pathologists to make an accurate diagnostic.

Characterising tumour molecular subtypes as well as the different infiltrating populations is also a decisive element in patient care. Thus, using the Avenio system, a link between the degree of intratumoral heterogeneity and the aggressiveness of metastases was revealed. Moreover, a highly selected event driving metastasis and increasing mortality was identified in clear-cell renal cell-carcinoma[105]. The combined use of manual microdissection and coring techniques has made it possible to redefine pancreatic ductal adenocarcinoma classification. To this end, the authors have identified the molecular characteristics of both the tumour microenvironment

and tumour cells. With five pancreatic ductal adenocarcinoma subtypes, this classification thus paves the way for more precise and effective treatment for this cancer[106].

Some studies have also sought to improve identification and isolation of specific populations by combining staining techniques such as immunohistochemistry or *in situ* hybridisation with microdissection[107]. Defined as expression microdissection[108]or immunoguided microdissection[109] these techniques have proven their value by showing enrichment of specific populations and associated markers in the selected samples[110,111]. The compatibility of these techniques with subsequent molecular analysis such as ddPCR or NGS has been demonstrated in KRAS mutated patients and PDX mouse tissue[111].

Finally, the combined use of microdissection and IHC for BRAFV600E detection on a few cancer cases has shown limitations. Mutation detection was highly dependent on the antibody used and its pre-processing conditions influenced the quality of DNA[112].

4.3. Proteomic approaches combined with microdissection

Proteins provide insight into biological mechanisms such as cancer and are therefore crucial for diagnosis. Their expression can vary significantly between different conditions and various processes such as post translational modifications can modify their biological characteristics and induce the emergence of pathological processes[113]. As with genetic and epigenetic processes, identifying the proteins involved in this process is crucial for understanding the disease.

Techniques such as gel electrophoresis require a large quantity of starting material to be effective. Combining these techniques with FFPE micro-samples contaminated with formalin and paraffin residues therefore seems to be a difficult task. It has been demonstrated that FFPE samples are much less suitable for Western blot techniques compared to fresh frozen and alcohol-fixed samples[114]. In this work, a minimum of 500 cells was necessary in FFPE

conditions to detect highly expressed proteins, thus highlighting the sensitivity limit of these techniques, especially with FFPE samples. No further works combining microdissection with FFPE samples and gel electrophoresis techniques were found, highlighting the limitations encountered.

However, combination with other more sensitive proteomics techniques such as mass spectrometry remains largely possible. Mass spectrometry is a powerful and sensitive technique widely used in oncology to identify a large number of peptides from a sample[115]. To make routine use of this technique possible in pathology practice, a high throughput protocol adapted to FFPE micro-samples has been described[116]. According to the authors, this workflow makes possible complete analysis of around a hundred samples in about ten days. Another optimisation work in mass spectrometry demonstrated the ability to work on H&E stained micro samples for studying spatial proteomic heterogeneity[117].

A variation of this technique, liquid chromatography coupled with tandem mass spectrometry (LC MS/MS) which has the advantage of being more specific thanks to prior separation steps, has been used in combination with the isolation of specific populations[118]. An optimised workflow allowing use of this technique on FFPE micro samples has been described by Longuespée et al.[119] The potential of this protocol was shown in triple negative breast cancer by revealing the presence of specific biomarkers differentiating invasive ductal carcinoma and invasive lobular carcinoma. In another study, LC MS/MS analysis of breast cancer stroma isolated with LPMC, was coupled with an analysis of the secretome of breast tumour cell lines. The results revealed a stromal protein base hypoxia profile that made it possible to distinguish between the different tumour subtypes to predict cancer aggressiveness and potential response to radiotherapy[120]. In anal cancer, this approach, combined with microdissection, identified two subpopulations of squamous cell carcinoma with specific protein signatures[121]. Finally, another

optimisation work has outlined a xylene-free extraction protocol for LC-MS/MS making it possible to work on cores smaller than 1mm^3 [122].

The matrix-assisted laser desorption ionisation (MALDI) technique is a powerful tool that gives access to spatial resolution of the whole tissue section without the need for an upstream dissection step as required by other MS techniques. Despite this, the low fragmentation yield provided here prevented optimal analysis of the tissues[123]. This system can therefore be seen as an image guiding tool for subsequent precise characterisation of tissue proteomes. Thus, a workflow combining MALDI and microdissection to study intratumor heterogeneity has been developed[124] and paired with LC-MS/MS analysis in invasive breast cancer as a proof of concept[125].

5) Discussion

The mechanisms that lead to the development of cancers are highly diverse and need to be identified to develop appropriate treatments. This task can prove challenging due to the high degree of heterogeneity in the populations and signals found within tumours[3,126]. The ability to isolate specific ROI or subpopulations may therefore provide a partial solution to this challenge and a variety of microdissection techniques have been developed to respond to this need.

FFPE samples are used for diagnosis and are stored and archived for decades. Experts estimate between 400 million and 1 billion FFPE tissues are stored in hospitals and biobanks around the world[127,128]. Consequently, these samples are an invaluable resource that could easily be used to decipher the mechanisms underlying diseases. Despite this, there are many technical constraints associated with these samples, forming a real obstacle for their use[128]. Most research studies focus on using fresh frozen tissue, considered the gold standard for tissue quality. The amount of work using microdissection on FFPE samples is therefore very limited. The aim of

this review was also to demonstrate the combined usability of microdissection and FFPE samples and to make using this approach possible on a larger scale in the near future.

The various dissection techniques developed each respond to specific needs. Where laser microdissection techniques facilitate isolation of pure cell populations in a time-consuming process, techniques such as mesodissection remove larger ROI with a higher flow rate (Table 1). The choice of microdissection techniques must be adapted to the user's constraints and expectations (Figure 3). Comparing milling and microfluidics technology with laser and manual microdissection has shown its value for fine selection of areas of interest for early signal detection[129]. In addition, this study showed similar sensitivity between mesodissection and laser microdissection. The logical link between increased accuracy and earlier detection has also been demonstrated in other works[130,131]**.** However, with the development of sensitive techniques, in some cases it is no longer necessary to obtain pure cell populations to detect specific mutations or subpopulations.

Based on these elements, techniques such as laser microdissection, which are time-consuming but more precise, are clearly more suited for basic research projects where the characterisation of pure populations is often necessary (Figure 3)

Isolating the different areas and sub-populations is of clear interest, as it not only increases the sensitivity of the tests carried out [130,131] but also makes it possible to work on small samples such as microbiopsies, obtained with core needle biopsy or fine needle aspiration[132]. In addition, the large number of cases analysed daily means that pathologists need fast, reliable and accessible systems. Recent developments in scraping methods using the Tissector (Xyall) and coring methods using the MiniPunch (Excilone) and TMA Molmed (3D Histech) technologies have met the needs of pathologists. In fact, the cutting precision of these systems is not comparable to those obtained with manual microdissection (Table 1). Like laser microdissection systems, these innovative systems provide full traceability of the operations,

but the main advantage associated with these techniques is their sampling flow rates (Table 1), which are much higher than those of any other technique, allowing them to be used routinely on a large scale (Figure 3).

Therefore, pathologists do not need to obtain pure populations for molecular diagnosis, and the reason why many of them do not perform such precise microdissection techniques is the absence of accessible and effective techniques. In daily practice, when tumour cell enrichment is not required, several sections (two to five, depending on the laboratory) are used, each 10 µm thick (Figure 3). These sections may be combined with manual macrodissection when tumour cell enrichment is required. This approach is sufficient to establish an accurate diagnosis in most cases. Microdissection techniques could be reserved for cases involving increased tissue complexity or microbiopsies, where the use of tissue must be rationalised (Figure 3). Thus, these technologies remain difficult for low-throughput laboratories to afford, primarily due to the substantial initial financial investment required. In addition, the ongoing costs of consumables (e.g. scraping heads and needles) and maintenance fees further limit accessibility. Furthermore, since microdissection is not systematically required, laboratories with low sample volumes often lack the incentive to invest in such technologies. These points suggest that microdissection techniques may become more widely used in high-throughput laboratories or specialised platforms (Figure 3).

Artificial intelligence (AI) is transforming the fields of pathology and oncology rapidly.[133,134] In this context, AI-based algorithms that can automatically identify and transfer ROI to microdissection platforms could represent the next major advancement in the field. Recently commercialised mesodissection systems (Xyall, MiniPunch and TMA Molmed) already integrate AI models that enable the automatic transfer of annotated ROIs from digital annotated slides to the corresponding slides or tissue blocks.

In diagnostic pathology, AI could be integrated with microdissection technologies and applied to routine techniques, such as immunohistochemistry (IHC). Following IHC staining, AI-based algorithms could be designed to identify and automatically transfer positively stained regions to the microdissection device. Numerous AI models have already been developed for tumour detection, segmentation and classification[13]. Integrating these models with microdissection systems could facilitate the isolation and characterisation of specific tumour cell populations.

In a basic research project, recent advances in high-resolution spatial transcriptomics technologies that are compatible with FFPE tissues have enabled us to gain unprecedented access to spatial information[13,13]. Incorporating AI-guided ROI identification, followed by isolation using a microdissection system, into such a workflow could refine the selection and characterisation of specific subpopulations further. Overall, combining AI with microdissection technologies is the next major step forward in this field. Achieving this successfully will greatly enhance the future implementation of microdissection in diagnostic and research practices.

The constant adaptation and innovation of analytical techniques to the problematics associated with FFPE and micro samples have made it possible to use microdissection as a routine analysis tool. Microdissection is such a promising field whose contribution to understanding the complex mechanisms associated with cancers, as well as many other pathologies, is not negligible. Now, with the era of personalised medicine, facilitated access to microdissection technologies can play a pivotal role in improving patient care.

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Abbreviation list: FF: freshly frozen, FFPE: formalin-fixed paraffin-embedded, ROI: region of interest, UV: ultraviolet laser, IR: infrared laser, LCM: laser capture microdissection, LMPC: laser

microdissection and pressure catapulting, CRC: colorectal cancer, NGS: next-generation sequencing, MIS-H: microsatellite-high, LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry, MALDI: matrix-assisted laser desorption ionization, AI : Artificial Intelligence, IHC : Immunohistochemistry.

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Figure legends

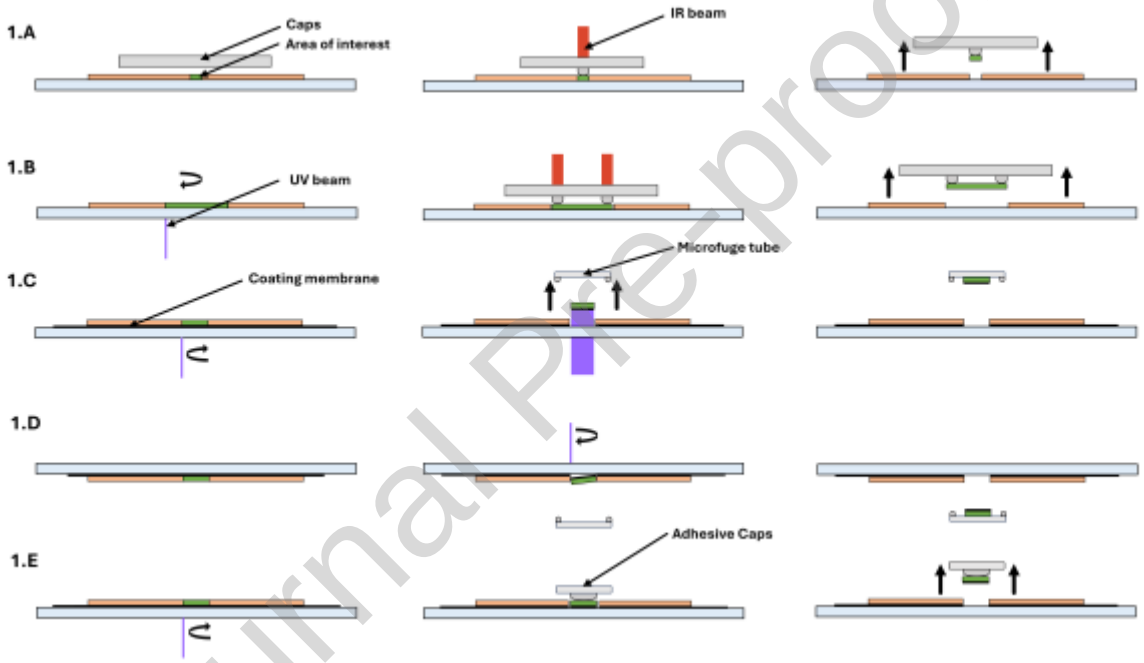

Figure 1: Laser Microdissection systems. (A) ***Laser capture microdissection systems.*** The cap containing the thermolabile membrane is first placed over the tissue previously mounted on a slide (Step 1). The microdissection device emits an IR beam that fuses the thermolabile membrane to the ROI using different beam diameters (7.5, 15 or 30 µm) (Step 2). The ROI fused to the membrane is then lifted from the surrounding tissue by the Cap moving vertically (Step 3)**. (B*) LCM system with UV and IR beam.*** The ROI is first isolated from the surrounding tissue with a UV beam emitted by the microdissection device (Step 1). A cap containing the thermolabile membrane is positioned above the isolated ROI. The membrane is then focused on the ROI at multiple points using the IR beam emitted by the microdissection device (Step

2). This process also allows the entire ROI to be removed in a single step while maintaining its structural integrity. Finally, the ROI is lifted from the slide via the Caps' vertical movement (Step 3). **(C)** ***Laser Microdissection and Pressure Catapulting system.*** The ROI is first cut out by a UV beam emitted by the microdissection device (Step 1). Samples are mounted on an energy transfer coating membrane, which enables the entire ROI to be irradiated by a single, high-energy, defocused UV beam, while preserving its structural integrity (Step 2). Catapulted samples are then collected directly into a microfuge tube (Step 3). **(D)** ***Microdissection by gravity.*** The tissue is first placed upside down and sandwiched between a energy transfer coating membrane and a metal frame (not shown here) (Step 1). The ROI is then isolated through the action of the UV beam emitted par the microdissection device (Step 2) and falls directly into a collection tube (Step 3). **(E)** ***Laser microbeam microdissection.*** The ROI is sandwiched between an energy transfer coating membrane and a metal frame (not shown here), and then it is isolated from the surrounding tissue by a UV beam emitted by the microdissection device (Step 1). The ROI is then isolated and captured using an adhesive insulation cap (Step 2). The system then lifts the ROI by the vertical movement of the adhesive insulation cap (Step3).

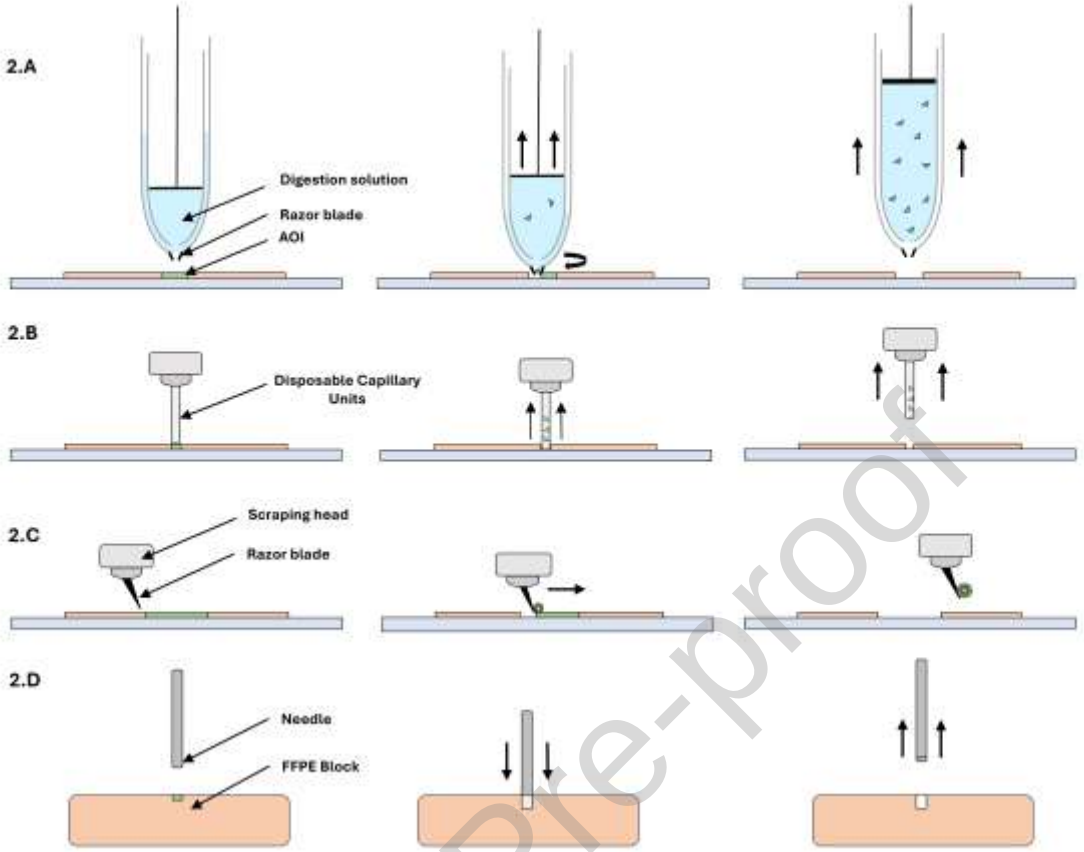

Figure 2: Mesodissection systems. (A) ***Milling and microfluidic system.*** The milling system, containing the enzymatic solution, is placed over the ROI (Step 1) and is then lysed via combined action of the blade and the enzymatic solution. The milling system incorporates a piston-based mechanism that enables the ROI to be sucked up and retrieved (Step 2). The mixture containing the lysed ROI and the enzymatic solution is then deposited into a dedicated tube (Step 3). **(B)** ***Capillarity system.*** The disposable capillary unit (DCU) system is placed over the ROI (Step 1) and will aspirate the ROI by capillarity (Step 2). The system is then removed from the slide and deposited in a dedicated tube (Step 3). **(C)** ***Scraping system.*** The scraping head containing the razor blade is positioned above the ROI. The blade is then brought into contact with the tissue, collecting the ROI by scraping it off (Step 2). The ROI collected on the razor blade is then deposited in a dedicated tube (Step 3). **(D)** ***Coring system.*** The needle is placed above the ROI directly on the block (Step 1). Then, the sample is taken by making a

punch directly on block (Step 2). The core and needle are then removed from the tube to recover the sample (Step 3).

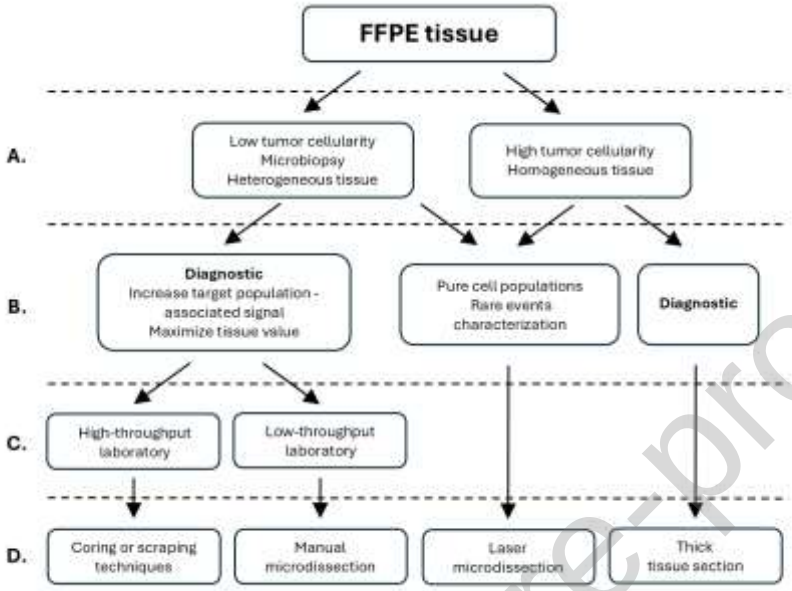

Figure 3: FFPE tissue handling decision flowchart. This decision flowchart guides the user in selecting the most appropriate microdissection technique. The first level is based on the biological characteristics of the tissue, including tumour cellularity and heterogeneity within the blocks. The amount of available biological material is also critical (**A**). The second level corresponds to the objectives of the experiment (**B**). Finally, the frequency with which these technologies are used, as well as the laboratory budget (**C**), also influence the choice of microdissection method **(D**).

Table 1: Main characteristics of various microdissection techniques

Technology		Accuracy	Flow rate	Suited use
Manual		>1 mm	Medium : Technician dependent	Isolation of large area without need of pure populations Basic research project Pathologist laboratory
Laser	IR	7.5-30 µm	Low :	

Mesodissection	UV	µm	Time consuming process	Isolation of pure population Basic research projects
	IR+UV	µm		
	Milling & microfluidics	250-750 µm	Medium : ± 9 min/sample[131]	Isolation of specific area without need of pure population Basic research project Pathology laboratory*
	Capillarity	10-100 µm	Medium : Several slides/hours	
	Scraping	100 µm	Fast : 30 or 80 slides/hours	
	Coring	200 µm	Fast : ± 1 min/sample	

**: For scraping and coring techniques. IR: Infrared, UV: Ultraviolet*

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Conflicts of interest and sources of fundings

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- M.G and Z.K are employees of Excilone, and P.D is its CEO and founder.
- For the remaining authors, none were declared.